(54) Title: METHOD OF ISOLATION OF DNA

(57) Abstract

A rapid and efficient method of DNA isolation, storage, and cleavage that provides DNA suitable for amplification by a sequence-specific method is provided. The method for isolating biologically active DNA from a biological sample having DNA-containing structures includes: (1) contacting a biological sample containing DNA-containing structures with a lysis and storage buffer comprising a non-amphipathic chaotropic salt sufficient to lyse DNA-containing structures in the sample and a chelating agent to preserve the DNA from degradation to form a mixture of the biological sample and the lysis and storage buffer; (2) incubating the mixture formed in step (1) with a metal-containing chemical nuclease that cleaves the DNA to DNA fragments; and (3) purifying the DNA fragments. The DNA isolated can be catenated closed circular DNA, such as kinetoplast DNA of *Trypanosoma cruzi*. The purified DNA fragments can be used for amplification in a sequence-based DNA amplification system employing primers that hybridize to the DNA in order to determine the presence of a specific DNA sequence in the fragments. The combination of the isolation and amplification methods can be useful for the detection of parasitic, bacterial, and viral diseases by identification of DNA sequences associated with the organisms causing them.

METHOD OF ISOLATION OF DNA

BACKGROUND

Increasingly, DNA-based diagnostic methods are
coming into use for the diagnosis of infectious and
parasitic diseases. Many of these techniques involve DNA
amplification procedures, such as the polymerase chain
5   reaction procedure (PCR), as described in United States
Patents Nos. 4,683,195, 4,683,202, 4,800,159, and
4,965,188 to Mullis et al. However, such DNA-based
amplification procedures require that DNA be isolated in
a stable, biologically active form from blood or other
10   biological fluids; optimal application of such
amplification procedures further requires that the DNA to
be amplified be present in the form of linear fragments
of less than about 2 kb in length. Moreover, because
there is often a considerable lapse of time between the
15   collection of a blood specimen from a patient and the
testing of DNA isolated from a blood sample, by DNA
amplification procedures, there is a need for a method of
storing DNA stably for periods of time in blood. There
further exists a need for the conversion of the DNA in
20   these biological samples, which may be in the form of
closed circular catenanes or other interlinked forms,
into linearized double-stranded DNA fragments suitable
for amplification. This method must be rapid, easy to

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perform, and capable of releasing the DNA efficiently
from cells or other structures and cleaving it into
fragments suitable for amplification.

5   The need for a method of isolation and storage
of DNA is particularly acute in connection with the
diagnosis of parasitic diseases by DNA amplification
procedures. Many of these diseases, including Chagas'
disease, leishmaniasis, and malaria, occur primarily in
10   tropical areas in which medical facilities are relatively
primitive and a substantial period of time may elapse
between collection of the sample from a patient suspected
of infection and its testing. Recently, considerable
work has been directed towards diagnosis of these
15   diseases by PCR, as described in Avila et al. (Mol.
Biochem. Parasitol. 42:175 (1990)), Sturm et al. (Mol.
Biochem. Parasitol. 33:205 (1989)), Rodgers et al. (Exp.
Parasitol. 71:267 (1990)) and Moser et al. (J. Clin.
Microbiol. 27:1477 (1989)). Additionally, Ashall et al.
20   (J.Clin. Microbiol. 26:576 (1988)) describe the detection
of Trypanosoma cruzi in mammalian blood by a DNA-DNA
hybridization procedure employing radiolabeled total
parasite DNA under conditions that favor hybridization of
repetitive DNA sequences.

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           The use of high EDTA concentrations to isolate
DNA has been described in Simpson & Berliner, "Isolation
of the Kinetoplast DNA of Leishmania tarentolae in the

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form of a Network," _J. Protozool._ 21:382-393 (1974).
Zolg et al. (_Am. J. Trop. Med. Hyg._ 39:33 (1988))
describe a method for storage of blood samples to be
tested for the presence of the parasite _Plasmodium_

5   _falciparum_ using specific DNA probes, in high salt
lysates. The lysates are prepared using water to lyse
the erythrocytes; a detergent/EDTA mix to lyse the
parasites, followed by the addition of cesium
trifluoroacetate. Disadvantages of this procedure

10   include the necessity to lyse the erythrocytes first, the
requirement for use of a detergent, and the expense of
the cesium salt. In addition, this system may inhibit
enzymes such as exonucleases added to the lysate to
cleave DNA.

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The need for a method to isolate and prevent
degradation of DNA in blood samples from patients
suspected of infection with parasites or other infectious
agents during storage, and to prepare the DNA for

20   amplification and/or hybridization procedures so that
diagnosis of infection can be accomplished, has gone
unmet.

SUMMARY

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Accordingly, the present invention provides a
rapid and efficient method of DNA isolation, storage, and
cleavage that meets these needs and provides DNA suitable

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for amplification by a sequence-specific method, such as
PCR. In general, the method of the present invention for
isolating biologically active DNA from a biological
sample having DNA-containing structures comprises the

5   steps of:

(1) contacting a biological sample containing
DNA-containing structures with a lysis and storage buffer
comprising a non-amphipathic chaotropic salt sufficient
to lyse DNA-containing structures in the sample and a

10   chelating agent to preserve the DNA from degradation to
form a mixture of the biological sample and the lysis and
storage buffer;

(2) incubating the mixture formed in step (a)
with a metal-containing chemical nuclease that cleaves

15   the DNA to form DNA fragments; and

(3) purifying the DNA fragments.

The DNA in the DNA-containing structures can be
present in the form of catenated closed circles. It can

20   be isolated from a parasite. In particular, the DNA can
be isolated from a kinetoplastid parasite such as
Trypanosoma cruzi. Alternatively, the DNA can be
associated with a virus such as herpes simplex virus
(HSV). RNA can also be isolated from RNA-containing

25   structures, and DNA can be produced from the RNA using
reverse transcriptase for amplification. The biological
sample can be a fluid sample such as mammalian blood,
including human blood.

The non-amphipathic chaotropic salt is preferably a guanidinium salt, such as guanidinium chloride or guanidinium thiocyanate. More preferably, the guanidinium salt is guanidinium chloride. Most preferably, the guanidinium chloride is present in at least about 3 molar concentration in the mixture of the biological sample and lysis and storage buffer.

The chelating agent is preferably ethylenediamine- tetraacetic acid (EDTA). Most preferably, the EDTA is present in at least about 0.1 molar concentration in the mixture of the biological sample and lysis and storage buffer.

The general method can further comprise the steps of:

(4) amplifying the DNA by a sequence-specific method employing at least two primers that hybridize to the DNA fragments; and

(5) detecting disease associated with the presence of the DNA in an organism by identifying the DNA.

The primers employed in the sequence-specific method can comprise at least two primers that hybridize to a genetic marker of the organism from which the biological sample was isolated for identification of the genetic marker.

The metal-containing chemical nuclease can be selected from 1,10-phenanthroline-copper complex, derivatives of ferrous EDTA, metalloporphyrins, or octahedral metal complexes of 4,7-diphenyl-1,10-

5 phenanthroline. Preferably, the metal-containing chemical nuclease is the 1,10-phenanthroline-copper complex.

An application of the general method is a

10 method for detecting a disease caused by a kinetoplastid parasite. This method comprises the steps of:

(1) contacting a biological sample from a patient suspected of having a disease caused by a kinetoplastid parasite, with a lysis and storage buffer

15 comprising a non-amphipathic chaotropic salt sufficient to lyse cells containing catenated closed circular kinetoplast DNA in the sample and a chelating agent to preserve the DNA in the cells from degradation to form a mixture of the biological sample and the lysis and

20 storage buffer;

(2) incubating the mixture obtained in step (1) with a metal-containing chemical nuclease to linearize catenated closed circular kinetoplast DNA to form kinetoplast DNA fragments;

25 (3) purifying the DNA fragments to form purified kinetoplast DNA fragments suitable for amplification;

(4) amplifying the purified kinetoplast DNA
fragments by a sequence-specific method employing at
least two primers capable of hybridizing to the
linearized DNA to form amplified kinetoplast DNA; and

5   (5) detecting disease by identifying the
presence of the amplified kinetoplast DNA corresponding
to the primers and thus having sequences specific for
kinetoplast DNA of the parasite.

10   When this method is used to detect Chagas'
disease, caused by _T. cruzi_, the primers hybridize to
conserved regions in _T. cruzi_ kinetoplast minicircular
DNA.

15   A preferred method according to the present
invention for the detection of a disease caused by _T._
cruzi comprises:

               (1) mixing a mammalian blood sample suspected
of containing kinetoplastid DNA from _T. cruzi_ with about
20   an equal volume of lysis and storage buffer comprising 6
M guanidinium chloride and 0.2 M EDTA, pH 8.0, to form a
mixture and storing the mixture at room temperature for
at least 24 hours;

               (2) incubating the mixture obtained in step (1)
25   with the metal-containing chemical nuclease 1,10-
phenanthroline-copper complex to cleave the kinetoplast
DNA to form linearized kinetoplast DNA;

(3) purifying the linearized kinetoplast DNA to form purified linearized kinetoplast DNA by:

(a) deproteinization with a phenol-chloroform mixture;

(b) ethanol precipitation with a glycogen carrier; and

(c) filtration through a microconcentrator;

(4) amplifying the purified linearized kinetoplast DNA by a sequence-specific method employing at least two primers capable of hybridizing to conserved regions of the catenated closed circular kinetoplast DNA of T. cruzi; and

(5) detecting disease caused by T. cruzi by identifying the presence of amplified DNA corresponding to the primers and thus having sequences specific for catenated closed circular kinetoplast DNA of T. cruzi by hybridization to the primers. Typically, the linearized DNA comprises fragments of from about 1 kb to about 1.5 kb in length.

The isolation and storage method steps of the method of the present invention can be practiced separately from cleavage of the DNA. A method of isolating and storing DNA from a cell-containing biological sample according to the present invention comprises the steps of:

(1) contacting a biological sample containing
DNA present in cells with a lysis and storage buffer in
order to liberate the DNA from the cells, the lysis and
storage buffer comprising:

5   (a) a concentration of a non-amphipathic
chaotropic salt sufficient to lyse cells in the
biological sample when the lysis buffer is contacted with
the biological sample; and

(b) a concentration of a chelating agent
10   sufficient to preserve the DNA from degradation when the
lysis buffer is contacted with the biological sample; and

(2)   storing the liberated DNA in the mixture
at a temperature of below about 65°C.

15   Preferably, the storage temperature is below
about 4°C in order to preserve the DNA sample for at
least about one year.

BRIEF DESCRIPTION OF THE DRAWINGS

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Figure 1 shows the results of testing the
stability of plasmid DNA as described in Example 1,
infra.

25   Figure 2 shows the results of cleavage of
isolated Trypanosoma cruzi kinetoplast DNA by the 1,10-
phenanthroline-copper chemical nuclease (OP-Cu^{2+}) as
described in Example 2, infra.

Figure 3 shows the frequency of single strand nicks in OP-Cu^{2+} linearized minicircle DNA as described in Example 2, _infra_.

Figure 4 shows results of a sensitivity titration of OP-Cu^{2+}-cleaved DNA, as described in Example 4, _infra_.

Figure 5 shows results from a blood sample of a chronic chagasic patient analyzed for the presence of _T. cruzi_ minicircle sequences, as described in Example 5, _infra_.

Figure 6 shows the results of PCR amplification of _T. cruzi_ DNA isolated from triatomid bug feces as described in Example 5, _infra_.

DESCRIPTION

The present invention is a rapid and efficient method of storing, isolating, and purifying biologically active DNA from a biological sample having DNA-containing structures. The DNA-containing structures include cells and viral particles. The method is particularly useful for isolating parasitic DNA that is present in the form of catenated closed circles, but the method is not limited to use on such DNA. The method allows for storage of the DNA in a state suitable for subsequent

controlled degradation by a chemical nuclease in order to
cleave the DNA to fragments of a size suitable for
subsequent amplification. The DNA can then be rapidly
purified to render it suitable for use in an

5   amplification method such as PCR.

       In general, the method comprises:
             (1) contacting a biological sample containing
       DNA-containing structures with a lysis and storage buffer

10   comprising a non-amphipathic chaotropic salt sufficient
       to lyse DNA-containing structures in the sample and a
       chelating agent to preserve the DNA from degradation and
       prevent coagulation of the blood to form a mixture of the
       biological sample and the lysis and storage buffer;

15   (2) incubating the mixture formed in step (1)
       with a metal-containing chemical nuclease that cleaves
       the DNA to DNA fragments; and
             (3) purifying the DNA fragments.

20   The method is particularly adapted for, but is
       not limited to, detection of parasitic diseases caused by
       parasites having catenated closed circular kinetoplast
       DNA, such as _Trypanosoma cruzi_, the protozoan causing
       Chagas' disease; (Sturm et al., _Mol. Biochem. Parasitol._

25   33:205 (1989); Avila et al., _Mol. Biochem. Parasitol._
       42:175 (1990); Moser et al., _J. Clin. Microbiol._ 27:1487
       (1989); Ashall et al., _J. Clin. Microbiol._ 26:576
       (1988)); _Leishmania_ species, the protozoa causing

leishmaniasis (Rodgers et al., _Exp. Parasitol._ 71:267

(1990)), and _Plasmodium falciparum_, _P. vivax_, and _P._

malariae, the protozoa causing human malaria (Zolg et

al., _Am. J. Trop. Med. Hyg._ 39:33 (1988)). In addition,

5   it can be used for the detection of other infectious

diseases and infectious agents such as the provirus of

human immunodeficiency virus (HIV), cytomegalovirus

(CMV), hepatitis B virus, herpesvirus, Epstein-Barr

virus, and _Toxoplasma gondii_.

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I.   ISOLATION OF DNA

     A.   The Biological Sample

15   The method is suitable for use on any

biological sample containing DNA. The method is

particularly adapted for storage and isolation of DNA

from mammalian blood, including human blood, but the

method is suitable for use on other biological samples,

20   such as insect feces, biopsy tissue, urine, sputum, and

lymphatic fluid. The method can be used on any volume of

fluid sample, from microliters to liters, as needed.

     B.   The Lysis and Storage Buffer

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           The biological sample is mixed with the lysis

and storage buffer of the invention. The lysis and

storage buffer comprises: (1) a concentration of a non-

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amphipathic chaotropic salt sufficient to lyse cells in the sample and (2) a concentration of a chelating agent sufficient to preserve the DNA from degradation. A non-amphipathic chaotropic salt is a salt, other than a

5   detergent having distinct polar and non-polar moieties, that disrupts non-covalent bonds, such as hydrogen bonds, salt links, hydrophobic interactions, and van der Waals interactions, that are primarily responsible for the maintenance of secondary, tertiary, and quaternary

10   structure in proteins and nucleic acids.

        The non-amphipathic chaotropic salt is preferably guanidinium chloride or a chemically related salt such as guanidinium thiocyanate; other chaotropic

15   salts such as lithium bromide, potassium thiocyanate, or potassium iodine are also usable. Most preferably, the guanidinium chloride is present in at least 3 molar concentration in the mixture of the biological sample and the lysis and storage buffer.

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        The chelating agent is preferably EDTA, although other chelating agents, such as sodium citrate, can also be used. Most preferably, the EDTA is present in at least 0.1 molar concentration in the mixture of the

25   biological sample and the lysis and storage buffer.

        Typically, the biological sample is mixed with about an equal volume of the lysis and storage buffer

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containing about 6 molar guanidinium chloride and about

0.2 molar EDTA, pH 8.0.

5   When the lysis and storage buffer is mixed with
the biological sample, the DNA-containing structures,
such as cells or viral particles, present in the
biological sample are lysed essentially instantaneously
and the DNA is liberated from the DNA-containing
structures. The DNA is stable in the mixture of the
10   biological sample and lysis and storage buffer and can be
stored at a storage temperature below about 65°C. At a
storage temperature of 37°C, DNA stored in a mixture of
lysis and storage buffer and sample remains intact for at
least a month. At 4°C or -20°C, the DNA is stable for at
15   least one year. The DNA can be stored at this stage for
subsequent cleavage with the chemical nuclease and
purification according to the remainder of the process of
the present invention, or may be used in other
techniques, such as nick translation to produce
20   hybridization probes.

       When the biological sample is blood, it is
preferred to store the mixture of blood and lysis and
storage buffer for an initial period of about 24 hours at
25   room temperature (i.e., about 20-25°C), followed by
subsequent storage at 4°C. It is not necessary to use
freshly drawn blood; blood stored for periods of up to
about 1 week can be used. In one version of the

procedure, blood is drawn into tubes containing

anticoagulants such as citrate and/or heparin and then

used after a period of storage of about 24 hours.

5   C.   The Chemical Nuclease

         The DNA in the lysis and storage buffer is

fragmented by incubation of the biological sample-lysis

and storage buffer mixture with a metal-containing

10   chemical nuclease capable of nicking DNA by oxidative

attack on the deoxyribose moiety (Sigman & Chen, Annu.

Rev. Biochem. 59:207 (1990)). The chemical nuclease can

be a 1,10-phenanthroline-copper complex (Sigman,

Biochemistry 29:9097 (1990)); a derivative of ferrous

15   EDTA such as methidiumpropyl-EDTA-iron (Hertzberg &

Devan, J. Am Chem. Soc. 104:313 (1982); Schultz et al.,

J. Am. Chem. Soc. 104:6861 (1982); Tullius & Dombroski,

Proc. Natl. Acad. Sci. U.S.A. 83:5469 (1986));

metalloporphyrins (Ward et al., Biochemistry 25:6875

20   (1986); Lee Doan et al., Biochemistry 25:6376 (1986);

Groves & Farrell, J. Am. Chem. Soc. 111:4998 (1989)) or

metal-containing octahedral complexes of 4,7-diphenyl-

1,10-phenanthroline such as tris (4,7-diphenyl-1,10-

phenanthroline) ruthenium (Barton, Science 233:727

25   (1986)). Preferably, the chemical nuclease is a 1,10-

phenanthroline-copper complex, because it is not

sensitive to inhibition by buffer components. In

addition, the 1,10-phenanthroline-copper complex is

inexpensive and its reaction can be efficiently quenched

by chelating agents in order to control the extent of

cleavage. Other chemical nucleases, such as ferrous-

EDTA, methidiumpropyl-EDTA-iron, metalloporphyrins, and

5   metal-containing octahedral complexes of 4,7-diphenyl-

1,10-phenanthroline, may be useful provided they are not

substantially inhibited by the buffer.

The phenanthroline-copper chemical nuclease

10   reagent introduces random single strand nicks into duplex

DNA in the presence of peroxide (Sigman & Chen, <u>Annu.</u>

<u>Rev. Biochem.</u> 59:207 (1990)). Because, on the average,

one double strand cleavage occurs after ten random single

strand breaks are introduced into a DNA molecule, this

15   nuclease can be used to digest catenated kinetoplast DNA

to linearize minicircles.

The chemical nuclease reaction using the 1,10-

phenanthroline-copper complex is typically performed as

20   follows: to one volume of the mixture of the biological

sample and lysis and storage buffer containing 3 molar

guanidinium chloride and 0.1 molar EDTA are added 0.1

volume each of 1 molar $MgCl_2$, 200 millimolar $CuSO_4$, 20

millimolar 1,10-phenanthroline and 7.5% H_2O_2 (freshly

25   diluted from 30% stock solution). The reaction is

initiated by addition of 0.1 volume of 3-

mercaptopropionic acid and digestion of DNA is allowed to

proceed for 30 minutes at 37° C. The reaction is stopped

by addition of 0.1 volume of 1.5 molar 2,9-dimethyl-1,10-phenanthroline. Reactions with other chemical nucleases are carried out as specified in the literature describing them. The cleavage process cleaves DNA to fragments that

5   can be used in an amplification process. These fragments are linearized and are typically of about 1 to about 1.5 kb length.

The 1,10-phenanthroline-copper complex is

10   believed to generate a highly reactive oxidative species and to react with the DNA through the formation of a reversible complex between the phenanthroline-copper reagent and the DNA. The reaction produces the following stable products: 5'-phosphorylated-termini, 3'-

15   phosphorylated-termini, free bases and 5-methylenefuranone, as well as minor amounts of 3'-phosphoglycolate termini. The predominant reaction involves initial oxidative attack at the C-1 hydrogen of the deoxyribose by the DNA-bound coordination complex.

20   Oxidative reaction is initiated within the minor groove of the DNA and the reagent exhibits preferential reactivity for DNA in the B form of the helix relative to DNA in the A form of the helix. The reaction is not specific for the nucleotide at the site of scission, but

25   its rate does depend on local sequence. The most important influence on the intensity of cutting by the phenanthroline-copper complex at any sequence position is the neighboring 5'-nucleotide. Because the reaction can

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be terminated at any time by the addition of a chelating

agent with high affinity for copper, such as 2,9-

dimethyl-1,10-phenanthroline, 2,9-dimethyl-4,7-

phenanthroline, or EDTA, compensations can be made for

5   any variations in reactivity due to local sequence.

Preferably, the chelating agent used to quench the

reaction is 2,9-dimethyl-1,10-phenanthroline. Thus, the

cleavage method described herein is essentially

independent of the DNA sequence, and the size of single-

10   stranded DNA fragments produced by cleavage using the

nuclease may be effectively controlled by regulating the

time of digestion using the chelating agent.

        In particular, cleavage with the 1,10-

15   phenanthroline-copper complex of _T. cruzi_ kinetoplast

DNA, which is originally present in the form of closed

circular catenated minicircles, results in individual

linear DNA molecules of about 1.4 kb in length.

20   D.   DNA Purification

        The DNA is preferably purified to remove

substances that can interfere with subsequent primer-

based DNA amplification. Purification typically

25   includes: (1) deproteinization; (2) precipitation of the

DNA; and (3) filtration. The product of purification is

suitable for amplification by PCR or another primer-based

amplification step.

The deproteinization of the sample is typically performed by extraction with phenol or a 1:1 mixture of phenol and chloroform, which denatures proteins and separates proteins from nucleic acid. Preferably, the

5   deproteinization is performed with extraction with a 1:1 mixture of phenol and chloroform.

Precipitation of the DNA is typically conducted using ethanol in the presence of a glycogen carrier and

10   is preferably carried out in the presence of about 80 μg/ml glycogen and 0.3 molar sodium acetate at room temperature.

The final purification step can be performed by

15   filtration through a microconcentrator. A suitable microconcentrator is a Centricon-100 microconcentrator manufactured by Amicon (Beverly, Massachusetts).

Subsequent to purification, the isolated and

20   purified DNA can be amplified in a system, as described below, or can be used for other purposes for which highly purified DNA fragments are used, such as the generation of hybridization probes or incorporation into cloning vectors.

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II. AMPLIFICATION OF ISOLATED DNA

          The purified and fragmented DNA can be
amplified by a sequence-specific method employing at
least two oligonucleotide primers that hybridize to the
DNA fragments. Such methods include, but are not limited
to, the polymerase chain reaction (PCR method) of Mullis
et al. as described in United States Patents 4,683,195,
4,683,202, 4,800,159, and 4,965,188 to Mullis et al., all
of which are herein incorporated in their entirety by
this reference. Preferably, the PCR method is performed
using a thermostable DNA polymerase such as the Thermus
aquaticus polymerase Taq I described in U.S. Patent
Number 4,889,818 to Gelfand et al., and incorporated
herein by this reference. Further details on the PCR
method are given in PCR Protocols (M.A. Innis et al.,
eds., Academic Press (1989)), incorporated herein by this
reference.

          Other sequence-specific nucleic acid
amplification systems employing primers are also known,
such as the transcription-based amplification system of
Gingeras et al. described in European Patent Application
No. 368906, and the similar system of Davey and Malek
described in European Patent Application No. 329822, both
incorporated herein by this reference. These systems
make use of an alternating cycle of amplification as DNA
and RNA employing a primer incorporating a promoter, a

DNA-dependent RNA polymerase such as bacteriophage T7 RNA

polymerase, and a RNA-dependent DNA polymerase or reverse

transcriptase.

5   In any of these methods, the product of the

amplification is a discrete linear DNA fragment or series

of discrete DNA fragments that can be separated by

electrophoresis on an agarose gel and identified by

hybridization methods such as "Southern blot"

10   hybridization following transfer of the DNA to

nitrocellulose filters or other filters. Useful

hybridization procedures are described in E.M. Sutter,

"Detection of Specific Sequences Among DNA Fragments

Separated by Gel Electrophoresis," J. Mol. Biol. 98: 503

15   (1975), incorporated herein by this reference. The

hybridization is typically carried out with radioactively

labeled DNA oligonucleotide probes; this serves to

identify specific sequences originally present in the DNA

used for amplification. If the probes are comprised of

20   DNA associated with a disease-causing agent, such as a

pathogenic bacterium, a DNA virus, or the DNA provirus of

a RNA virus, the hybridization procedure can be used to

identify DNA sequences associated with the disease-

causing agent in the original sample and thus the

25   presence of the disease.

III. APPLICATIONS OF THE METHOD

        A.   Diagnosis Of Parasitic Diseases

5   The DNA storage, cleavage, purification, and
amplification method of the present invention is useful
for the detection of parasitic diseases, particularly
diseases caused by kinetoplastid trypanosomes.

10   These diseases include Chagas' disease, a major
health problem in Latin America caused by _Trypanosoma_
cruzi. Current serological and xenodiagnostic methods
for the diagnosis of Chagas' disease are unreliable,
especially in the case of chronic Chagas' disease in
15   which the parasites are detectable within the blood only
with difficulty, if at all, because of the low
concentration of parasites within the blood.

        T. cruzi kinetoplasts, which are DNA-containing
20   structures in the mitochondria of the cells, contain DNA
in the form of catenated minicircles, that, when cleaved
by the chemical nuclease and purified according to the
method of the present invention, can be efficiently
amplified by the PCR process or another sequence-specific
25   primer-based amplification process. Oligonucleotide
primers specific to conserved regions in _T. cruzi_
kinetoplast minicircles (kDNA) are used (Degrave et al.,
Mol. Biochem. Parasitol. 27:63-70 (1988); Sturm et al.,

Mol. Biochem. Parasitol. 33:205 (1989); Avila et al.,

Mol. Biol. Parasitol. 42:175 (1990)). These primers may

be synthesized and are also commercially available from

AMAC, Inc. (a Division of Genset, located in Westbrook,

5   ME).

     This results in a set of amplified DNA

fragments that are species-dependent and strain-

independent and can be used to detect the presence of T.

10   cruzi DNA in the blood of patients, as well as in

biological samples obtained from infected insect vectors

or infected mammals such as mice.

     In general, DNA isolated and amplified as

15   described herein may be used to detect disease associated

with the presence of the DNA using standard methods, such

as by DNA hybridization using radioactively labeled

probes to bind to specific sequences in the DNA (Sturm et

al., Mol. Biochem. Parasitol. 33:205 (1989) and Avila et

20   al., Mol. Biochem. Parasitol. 42:175 (1990)).

     B.   Other Applications and Advantages

     The method of the present invention is not

25   limited to storage, purification or detection of

parasitic DNA or to minicircular DNA. Because the

chemical nuclease that is employed typically cleaves DNA

into linear fragments of from about 1 kb to about 1.5 kb

in length, an optimal size for amplification with primers
of less than 1000 bases as required for PCR, the lysis,
storage, cleavage, and purification process of the
present invention can be used to detect DNA specific for

5   any biological entity of interest from any biological
sample, including blood, urine, sputum, or lymphatic
fluid. The DNA detected can be from any etiological
agent, including the DNA of parasitic protozoans,
bacteria, or viruses such as herpes viruses,

10   cytomegalovirus (CMV), hepatitis B virus, herpes virus,
Epstein-Barr virus, or Toxoplasma gondii. Cellular DNA
can also be detected, including mammalian or human DNA,
and retroviral DNA such as the DNA of human
immunodeficiency virus (HIV) associated with AIDS. The

15   amplification allows the detection of extremely small
quantities of DNA specific to a etiological agent, and
thus can be used to detect the presence of the disease
caused by the agent earlier than conventional serological
diagnostic procedures.

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       When whole blood is used for the isolation of
DNA according to the method of the present invention,
genetic markers of the host species (animal or human) can
be assayed in the same multiplex PCR assay in addition to

25   genetic markers of parasites or other etiological agents
of diseases. For example, in the case of humans, the
genetic markers assayed can include a family of immune
response genes such as the major histocompatibility locus

(MHC) genes which have been shown to be correlated with resistance to various diseases and susceptibility to autoimmune diseases. Other appropriate genetic loci for assay in multiplex PCR include the human homologues of

5   those genetic markers found in animal models to confer resistance to parasitic diseases such as those caused by Leishmania or Trypanosoma cruzi. Any desired genetic locus, such as oncogenes and anti-oncogenes or genes involved in genetic diseases can be targeted for

10   selective amplification during this assay, using the appropriate selection of primers and the isolated DNA.

       RNA can also be isolated from RNA-containing structures such as cells or viruses and stored in a

15   mixture of the sample and the lysis and storage buffer. The RNA can then be transcribed by reverse transcriptase to generate a RNA-DNA hybrid. The RNA in the hybrid is then degraded by the enzyme ribonuclease H specific for the RNA strand of a double-stranded RNA-DNA hybrid, and

20   the resulting DNA strand made double-stranded by hybridization of a suitable primer and elongation of the primer by DNA polymerase.

       An additional advantage of the method of the

25   invention is that the use of the lysis and storage buffer and chemical nuclease of the invention can contribute to reduction of the infectivity of endogenous infectious organisms such as viruses or bacteria present in the

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biological sample, because the infectivity is
substantially destroyed by lysis of the bacterial cells
or virus particles and also by cleavage of the viral
nucleic acid. Treatment of membrane enveloped viruses

5   with the 1,10-phenanthroline-copper chemical nuclease
destroys their infectivity (Lembech et al., <u>Fed. Proc.</u>
<u>44</u>:1072 (1985).

The invention is illustrated by the following

10   examples. The examples are for illustrative purposes
only and are not to be construed as limiting the scope of
the invention in any manner.

<u>Example 1</u>

15   <u>Storage Of DNA In Guanidinium Chloride-EDTA Buffer</u>

Human intravenous blood was freshly drawn and
collected into tubes containing an equal volume of 2 X GE
lysis and storage buffer (6 M guanidinium chloride, 0.2 M

20   EDTA, pH 8.0). The resulting GEB
(guanidinium/EDTA/blood) lysate was stored at 25°C for at
least 24 hours and subsequently at 4°C for up to 6
months.

25   To test the stability of DNA in GEB lysates,
plasmid DNA, pGEM 7Z (Promega, Madison, Wisconsin), was
added to 2 tubes of GEB lysates. Each tube was stored at
either 37°C or 65°C. Equivalent aliquots from each tube

27

were taken at different time intervals for up to four

weeks. The aliquots were extracted once with

phenol/chloroform (1:1, v/v) and ethanol-precipitated.

The isolated DNA was electrophoresed in a 1% agarose gel

5   to determine the percentage of nicked or linearized

plasmid DNA, which can be detected by its relatively

rapid mobility through the gel in contrast to intact

catenanes, which remain at the top of the gel.

10   The results are shown in Figure 1; the

electrophoretic pattern resulting from incubation at 37°C

is designated (1A) and the pattern resulting from

incubation at 65°C is designated (1B). The control lane

is unincubated plasmid DNA in 10 mM Tris-HCl, pH 8.0, 1

15   mM EDTA (TE). Figure 1 shows that the DNA remains intact

at 37°C for at least a month, with no apparent nicking or

degradation. At 65°C, the DNA is nicked after a two-week

incubation, as indicated by the disappearance of the

closed circular DNA band and the increase in the nicked

20   circular band. Even at 65°C, at least 50% of the DNA

remained in the nicked circular or linear form after one

week incubation. At 4°C or -20°C, DNA was shown to be

stable in GEB lysate for at least one year. These

results clearly demonstrate that the guanidinium

25   chloride-EDTA reagent is a suitable medium for the lysis

of cells contained in blood and the preservation of DNA

at room temperature for a substantial period of time.

28

Example 2

Cleavage Of Kinetoplast DNA

Using 1,10-Phenanthroline-Copper Complex

5   To 0.5 ml GEB lysate containing kinetoplast

DNA, 0.05 ml of each of the following solutions was

added: 1 M $MgCl_2$, 200 mM $CuSO_4$, 20 mM 1,10-phenanthroline,

and 7.5% H_2O_2 (diluted fresh from 30% stock). The

reaction was initiated by addition of 0.05 ml of 58 mM 3-

10   mercaptopropionic acid. Digestion of DNA was allowed to

proceed for 30 minutes or 60 minutes at 37°C. The

reaction was stopped by addition of 0.05 ml of 1.5 M 2,9-

dimethyl-1,10-phenanthroline. For GEB lysates containing

T. cruzi kinetoplast DNA digestion was carried out for 60

15   minutes at 37°C, and aliquots were removed every 10

minutes. The reaction was quenched by addition of 2,9-

dimethyl-1,10-phenanthroline. The aliquots were

deproteinized with phenol/chloroform (1:1) and

precipitated with ethanol. The DNA was denatured in

20   glyoxal/DMSO and loaded onto a 1% agarose gel and

electrophoresed as described by McMaster and Carmichael

(Proc. Natl. Acad. Sci. U.S.A. 74:4835 (1977)). The gel

was blotted and the DNA transferred to a Nytran filter

(Schuell and Schuster, Keene, New Hampshire). Total T.

25   cruzi kinetoplast DNA was nick-translated with ^{32}P-ATP and

used as a hybridization probe. Nick-translation and

hybridization conditions were as described in Simpson et

al., <u>Nucl. Acids Res.</u> 13:9577 (1985)), incorporated by

reference herein.

5   The extent of cleavage of purified <u>T. cruzi</u>

kinetoplast DNA in Tris-EDTA buffer (10 mM Tris-HCl, pH

8.0, 1 mM EDTA) by the phenanthroline-copper chemical

nuclease reagent was monitored by agarose gel

electrophoresis on a 1% agarose gel as a function of

digestion time (Figure 2). In Figure 2, the M lane shows

10   <u>Hind</u> III fragments of bacteriophage λ DNA and <u>Hae</u> III

fragments of bacteriophage φX174 RF DNA as size markers.

The disappearance of the kDNA from the well and the

appearance of the 1.4-kb linear minicircle band as a

function of digestion time is seen. After 10 minutes of

15   incubation, the DNA was completely cleaved to

minicircles.

        Undigested catenated kinetoplast DNA remained

in the well because, as a result of its large size, it

20   was unable to enter the gel. After one minute of

digestion, no catenated kinetoplast DNA could be detected

in the well and the released linearized minicircle DNA

migrated as a 1.4 kb band (Figure 2). Continued

digestion led to the appearance of a lower molecular

25   weight smear resulting from multiple cleavages per

minicircle molecule. However, the reaction could be

terminated at any point by the addition of a copper

30

chelating agent, such as 2,9-dimethyl-1,10-phenanthroline.

　　　　　If the duplex minicircle DNA fragments released

5　　from the catenated network contain excessive single

strand nicks, the DNA will not be an adequate substrate

for PCR amplification. Upon denaturation, the size of

the single-stranded fragment would have to be at least

equal to the distance between the two PCR primers in

10　　order to obtain successful amplification. As described

above, the DNA was cleaved with the phenanthroline-copper

chemical nuclease in GEB lysate for increasing periods of

time and the cleaved DNA was electrophoresed in a

denaturing glyoxal gel to determine the size distribution

15　　of single-stranded fragments. The gel was blotted onto a

nylon membrane and hybridized with ^{32}P-labeled T. cruzi

kinetoplast DNA (kDNA). At time 0, a few decatenated

minicircles undergoing replication can be seen as a 1.4-

kb band in the gel. After cleavage, the minicircles were

20　　released from the kDNA networks as double stranded

linearized molecules. By denaturing the linearized

minicircles, it can be seen that the minicircles were

increasingly nicked as a function of digestion time.

Figure 3 shows that with increasing incubation time there

25　　was a decrease in the size of the single-stranded

fragments caused by nicking. However, after 30 minutes,

90% of the minicircle fragments were larger than 310

bases. After 60 minutes, 50% of the fragments were

larger than 310 bases, and 80% of the fragments were

larger than 118 bases. An incubation time of 30 minutes

was selected for routine phenanthroline-copper digestion

of blood lysates, at which time approximately 90% of the

5   single-stranded fragments were longer than 310 bases, and

would be appropriate amplification target molecules for

the three sets of PCR primers which yield products of 83

bp, 122 bp, and 330 bp respectively (Sturm et al.,

supra). A control experiment showed the kinetoplast DNA

10   digested with the phenanthroline-copper reagent in GEB

lysate under standard conditions for 30 minutes was a

suitable template for PCR amplification.

Example 3

Isolation Of Cleaved Minicircle DNA

15

After GEB lysates of T. cruzi were digested

with the phenanthroline-copper reagent (OP-Cu^{2+}) for 30

minutes and the reaction quenched as described above, 500

20   μl aliquots containing decreasing numbers of minicircle

molecules (calculated from the DNA concentration) were

removed. Each 500 μl aliquot was extracted once with 100

μl phenol/chloroform (1:1). The aqueous phase was

transferred to an Eppendorf tube containing 40 μg of

25   glycogen (2μl of 20 mg/ml stock solution) and 50 μl of 3

M NaOAc. One milliliter of ethanol was added and the DNA

was precipitated at room temperature by spinning the

tubes in a microcentrifuge for 20 minutes.

32

The pellet was resuspended in 1 ml of water and
transferred to a Centricon-100 microconcentrator (Amicon)
containing 1 ml of water. The microconcentrator unit was
centrifuged at 1000 x g in a clinical centrifuge for 10

5   minutes. The retentate was washed a second time with 2
ml of water. After the second 10 minute centrifugation
100 μl of concentrated retentate was collected as
described by the manufacture of the microconcentrator.
The retentate was used for PCR amplification as described

10   below.

Example 4

Polymerase Chain Reaction Amplification

Of Cleaved Minicircle DNA

15

70 μl of the retentate material was amplified in a
100 μl PCR reaction. The reaction conditions were as
follows: 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 5 mM MgCl$_2$,
0.1 mg/ml BSA, 3 units of Taq DNA polymerase (Perkin-

20   Elmer Cetus) and 100 picomoles of each primer. The
primers used were three sets of primers specific to the
four conserved regions in T. cruzi minicircles yielding
the 330 bp minicircle variable and conserved region DNA
fragment and the 83 bp and 122 bp conserved region

25   fragments respectively, as described by Sturm et al.,
Mol. Biochem. Parasitol. 33:205 (1989) and Avila et al.,
Mol. Biochem. Parasitol. 42:175 (1990), both of which are
incorporated by reference herein. The combination of the

26 base primer S33A and the 26 base primer S34A resulted

in the generation of the 83 bp conserved region fragment.

The combination of S34A and the 26 base primer S67

resulted in the generation of the 122 bp conserved region

5   fragment. The combination of the 26 base primer S35 and

the 21 base primer S36 resulted in the generation of the

330 bp variable region fragment (Sturm et al., supra).

These primers are commercially available as Genset from

Amac, Inc., Westbrook, Maine. The cycling profile was as

10   follows: Denaturation, annealing, and elongation were

done at 94°C, 60°C, and 72°C, respectively; each step was

allowed to proceed for one minute for a total of 30

cycles. A 15 μl aliquot from each reaction was analyzed

on agarose/Nusieve (FMC, Rockland, Maine) gels.

15

To determine the sensitivity of the PCR-

minicircle DNA assay, the equivalent of 20 T. cruzi

kinetoplast DNA catenated networks were added to 10 ml

GEB lysate. The network DNA was cleaved with

20   phenanthroline-copper as described in Example 2. After

cleavage, the GEB lysate was diluted with kinetoplast

DNA-free GEB lysate to give DNA concentrations of from 1

to 10,000 minicircles for 500 μl of GEB lysate. Total

DNA was isolated from these aliquots as described in

25   Example 3 and subjected to PCR amplification; 1/6 of each

PCR reaction was loaded onto a gel. In all cases, the M

lane shows Hae III fragments of bacteriophage ϕX174 RF

DNA as size markers. In some cases, results are shown

from hybridization with a radioactively labeled probe after blotting of the gel as described in Example 2.

Figure 4A shows results from amplification of 83-bp fragments using 35 PCR cycles; the products were electrophoresed on a 2% agarose/3% Nusieve gel. The rapidly migrating band present in all lanes represents PCR primers or primer dimers.

Figure 4B shows results from amplification of 122 bp fragments using 30 PCR cycles; the products were electrophoresed on a 1% agarose/3% Nusieve gel. The top panel shows the stained gel; the low molecular weight band present in all lanes represents PCR primers. The bottom panel shows the hybridization of the blot with ^{32}P-labeled S34A oligonucleotide internal probe (Sturm et al., _supra_).

Figure 4C shows results from amplification of 330 bp variable and conserved region fragments with 30 PCR cycles; the products were electrophoresed on a 1% agarose/3% Nusieve gel. The top panel shows the stained gel. The bottom panel shows results from hybridization of the blot with ^{32}P-labeled S67 oligonucleotide internal probe (Sturm et al., _supra_; Avila et al., _Mol. Biochem._ _Parasitol._ 42:175 (1990)). The C1 and C2 lanes show 500 μl of undigested GEB lysate sample containing 10 kDNA

networks per 30 ml processed for PCR amplification as
described for all other samples.

As shown by the electrophoresis patterns of
5   Figure 4, at least 100 minicircles in 500 μl of GEB
lysate were detected, either by ethidium bromide staining
(83 bp PCR product) or by hybridization to a ^{32}P-labeled
oligonucleotide probe (122 bp and 330 bp PCR products).
Therefore this method detects the equivalent of 1% of the
10   minicircle content of a single T. cruzi cell in 500 μl of
phenanthroline-copper digested GEB lysate. These results
indicate that the method can be used for the detection of
a single T. cruzi cell in
20 ml of blood.

15

Example 5

Diagnosis of Chagas' Disease

A 49 year old female patient from Ecuador
20   showing cardiac disturbances tested positive for T. cruzi
in two different serological tests -- xenodiagnosis was
negative. The serological tests employed were complement
fixation (Sommerwirth and Jarett, Gradwohl's Clinical
Laboratory Methods and Diagnosis (C.V. Mosby, St. Louis,
25   1980)) and ELISA (Goldsmith & Heyneman, Tropical Medicine
and Parasitology (1989)). Xenodiagnosis was performed by
the following procedure (E.L. Segura, Xenodiagnosis. In
Chagas' Disease Vectors (Brenner and Stoka, eds., CRC

Press, Boca Raton, Florida, 1987), vol. II, pp. 41-45):

Uninfected triatomids of several species, including

Triatoma dimidiata, T. protracta and Rhodnius prolixus,

were allowed to feed upon the patient. Second, third,

5   fourth, and fifth nymphal as well as adult stages were

used. Approximately one-third of the bugs fed, and

defecation spots were found on the patient. Microscopic

examination of bug feces at 30 and 60 days after feeding

revealed no T. cruzi.

10

A 10 ml sample of venous blood from the patient

was obtained and stored as GEB lysate. Phenanthroline-

copper cleavage of the lysate was performed and DNA was

isolated from two 500 μl aliquots and PCR amplified as

15   described in Examples 2 through 4, supra.

Figure 5 shows a specific amplification of T.

cruzi minicircle sequences from patient blood with two

different sets of PCR primers. Figure 5A shows the

20   amplification of an 83 bp fragment; the products are

analyzed on a 1% agarose/3% Nusieve gel. The negative

control in lane 1 is a GEB sample from a non-chagasic

donor. The negative control in lane 2 is a sample

lacking kDNA in the PCR reaction. The M lane shows DNA

25   Hae III fragments of ϕX174 RF DNA as size markers.

Figure 5B shows the amplification of 330 bp variable and

conserved region fragments, run on a 2% agarose gel,

blotted and hybridized with ^{32}P-labeled S67

oligonucleotide internal probe (Sturm et al., <u>supra</u>;

Avila et al., <u>supra</u>.) A positive control is 100 fg gel-

isolated OP-Cu^{2+}-cleaved kDNA; the negative controls are

the same as (A).

5

Blood samples from four additional serology-

positive chronic chagasic patients (three patients were

xenodiagnosis-negative and one xenodiagnosis-positive)

also tested positive for <u>T. cruzi</u> parasites by PCR

10   amplification of minicircle DNA using the standard

procedure of Examples 1-4. These results indicate that

the method should prove useful in diagnosis of chronic

Chagas' disease.

15   The DNA storage, cleavage, purification, and

amplification method was extended to an insect vector and

to biopsy material from infected mice. The abdominal

contents of two <u>T. dimidiata</u> and two <u>R. prolixus</u> were

collected and stored in GE buffer. The samples were

20   processed as described in Examples 1-4. Figure 6 shows a

specific amplification of kinetoplast DNA minicircle

sequences from the insect abdominal contents.

Minicircle variable region fragments of 330 bp were

amplified. The PCR reactions were of 100 μl total

25   volume; 15 μl was loaded on a 2% agarose gel. The M lane

shows DNA <u>Hae</u> III fragments of ϕX174 RF DNA as size

markers. Positive and negative controls are 1 pg kDNA

and no kDNA, respectively, in the PCR reaction.

Animal biopsy material was also dissolved and
stored in GE. Heart tissue obtained from infected and
uninfected mice was washed with saline solution and
stored in GE buffer. The tissue was dissolved by

5   incubation in GE at 37°C for two days with occasional
vigorous mixing. The dissolved tissue was processed as
described in Examples 1-4. Specific PCR amplification of
minicircle sequences was observed from the heart tissue
lysates of the infected mouse but not from uninfected

10   controls. These results indicate that the method could
be used for autopsy specimens to detect Chagas' disease.

                 ADVANTAGES OF THE INVENTION

15   The method of the present invention has a wide
variety of potential applications in the diagnosis of
bacterial, parasitic, and viral diseases. It is
particularly useful for the collection of specimens from
patients in the field, in clinics and under other

20   conditions in which storage conditions may be less than
optimal. It is particularly adapted to the diagnosis of
parasitic diseases caused by kinetoplastid trypanosomes,
such as T. cruzi, the causative agent of Chagas' disease,
because the catenated kinetoplast minicircle DNA

25   characteristic of this organism is efficiently cleaved to
linear fragments of a size suitable for amplification by
a sequence-specific primer-based amplification technique
such as PCR. The method of the invention is simple to

carry out, rapid, and highly effective in detecting small

quantities of DNA specific to infectious agents. In

addition, the infectivity of biological samples

containing infectious agents is reduced by application of

5   the method of the invention.

Although the present invention has been

described in considerable detail with reference to

certain preferred versions thereof, other versions,

10   modifications and variations of the invention as set

forth above may be made without departing from the spirit

and scope of the present invention. Therefore, the

invention is limited only by the terms of the appended

claims.

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We claim:

1.　A method for isolating biologically active
DNA from a biological sample having DNA-containing

5　　structures comprising:

(a) contacting a biological sample containing
DNA-containing structures with a lysis and storage buffer
comprising a non-amphipathic chaotropic salt sufficient
to lyse DNA-containing structures in the sample and a

10　　chelating agent to preserve the DNA from degradation to
form a mixture of the biological sample and the lysis and
storage buffer;

(b) incubating the mixture formed in step (a)
with a metal-containing chemical nuclease that cleaves

15　　the DNA to form DNA fragments; and

(c)　purifying the DNA fragments.

2.　The method of claim 1 wherein the DNA in
the DNA-containing structures is present in the form of

20　　catenated closed circles.

3.　The method of claim 2 wherein the DNA is
DNA associated with a parasite.

25　　　　4.　The method of claim 3 wherein the parasite
is a kinetoplastid parasite.

5.   The method of claim 4 wherein the parasite
is _Trypanosoma cruzi_.

6.   The method of claim 1 wherein the chemical

5   nuclease is selected from the group consisting of 1,10-
phenanthroline-copper complex, derivatives of ferrous
EDTA, metalloporphyrins and octahedral metal complexes of
4,7-diphenyl-1,10-phenanthroline.

10   7.   The method of claim 6 wherein the chemical
nuclease is 1,10-phenanthroline-copper complex.

8.   The method of claim 7 wherein cleavage of
the nuclease is stopped by a copper chelating agent to

15   control the size of said DNA fragments.

9.   The method of claim 1 wherein the
biological sample is mammalian blood.

20   10.   The method of claim 9 wherein the
mammalian blood is human blood.

11.   The method of claim 1 wherein the non-
amphipathic chaotropic salt is a guanidinium salt

25   selected from the group consisting of guanidinium
chloride and guanidinium thiocyanate.

12. The method of claim 11 wherein the guanidinium salt is guanidinium chloride.

13. The method of claim 12 wherein the

5 guanidinium chloride is present in at least about 3 molar concentration in the mixture of the biological sample and lysis and storage buffer.

14. The method of claim 1 wherein the

10 chelating agent is EDTA.

15. The method of claim 14 wherein the EDTA is present in at least about 0.1 molar concentration in the mixture of the biological sample and lysis and storage

15 buffer.

16. The method of claim 1 further comprising the step of:

(d) amplifying the DNA by a sequence-specific

20 method employing at least two primers that hybridize to the DNA fragments.

17. The method of claim 16 further comprising the step of:

25 (e) detecting disease associated with the presence in an organism of the DNA subjected to amplification by identifying the DNA.

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18. The method of claim 17 wherein the DNA is identified using DNA hybridization.

19. The method of claim 1 wherein the DNA encodes a genetic marker of the organism from which the biological sample was isolated.

20. The method of claim 1 wherein the DNA is associated with a virus.

21. A method for detecting a disease caused by a kinetoplastid parasite comprising the steps of:

(a) contacting a biological sample from a patient suspected of having a disease caused by a kinetoplastid parasite, with a lysis and storage buffer comprising a non-amphipathic chaotropic salt sufficient to lyse cells in the sample and a chelating agent to preserve the DNA in the cells from degradation to form a mixture of the biological sample and the lysis and storage buffer;

(b) incubating the mixture obtained in step (a) with a metal-containing chemical nuclease to linearize catenated closed circular kinetoplast DNA to form kinetoplast DNA fragments;

(c) purifying the DNA fragments to form purified kinetoplast DNA fragments suitable for amplification;

(d) amplifying the purified kinetoplast DNA
fragments by a sequence-specific method employing at
least two primers capable of hybridizing to the
linearized DNA to form amplified kinetoplast DNA; and

5   (e) detecting disease by identifying the
presence of the amplified kinetoplast DNA corresponding
to the primers and thus having sequences specific for
kinetoplast DNA of the parasite.

10   22.   The method of claim 21 wherein the
biological sample is mammalian blood.

           23.   The method of claim 22 wherein the
mammalian blood is human blood.

15

           24.   The method of claim 21 wherein the non-
amphipathic chaotropic salt is a guanidinium salt
selected from the group consisting of guanidinium
chloride and guanidinium thiocyanate.

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           25.   The method of claim 24 wherein the
guanidinium salt is guanidinium chloride.

           26.   The method of claim 21 wherein the
25 chelating agent is EDTA.

           27.   The method of claim 21 wherein the
chemical nuclease is selected from the group consisting

of 1,10-phenanthroline-copper complex, derivatives of

ferrous EDTA, metalloporphyrins, and octahedral metal

complexes of 4,7-diphenyl-1,10-phenanthroline.

5   28.   The method of claim 27 wherein the

chemical nuclease is the 1,10-phenanthroline-copper

complex.

             29.   The method of claim 21 wherein the

10   kinetoplastid parasite is <u>Trypanosoma cruzi</u>.

             30.   The method of claim 21 wherein the primers

hybridize to conserved regions in <u>T. cruzi</u> kinetoplast

minicircular DNA.

15

             31.   A method for isolating and storing DNA

from a cell-containing biological sample comprising the

steps of:

             (a) contacting a biological sample containing

20   DNA present in cells with a lysis and storage buffer in

order to liberate the DNA from the cells, the lysis and

storage buffer comprising:

             (i) a concentration of a non-amphipathic

chaotropic salt sufficient to lyse cells in the

25   biological sample when the lysis buffer is contacted with

the biological sample; and

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(ii) a concentration of a chelating agent

sufficient to preserve the DNA from degradation when the

lysis buffer is contacted with the biological sample; and

(b)   storing the liberated DNA in the mixture

5   at a temperature of below about 65°C.

32.   The method of claim 31 wherein the non-

amphipathic chaotropic salt is guanidinium chloride

present in at least about 3 molar concentration in the

10   mixture of the biological sample and the lysis and

storage buffer and the chelating agent is EDTA present in

at least about 0.1 molar concentration in the mixture of

the biological sample and the lysis buffer.

15   33.   The method of claim 31 wherein the storage

temperature is below about 4°C in order to preserve the

DNA for at least about one year.

34.   The method of claim 31 wherein the DNA is

20   present in the cells in the form of catenated closed

circles.

35.   A method for detecting a disease caused by

the kinetoplastid parasite _T. cruzi_ comprising the steps

25   of:

(a) mixing a mammalian blood sample suspected

of containing kinetoplastid DNA from _T. cruzi_ with about

an equal volume of lysis and storage buffer comprising 6

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molar guanidinium chloride and 0.2 molar EDTA, pH 8.0 to

form a mixture and storing the mixture at room

temperature for at least 24 hours;

(b) incubating the mixture obtained in step (a)

5   with the chemical nuclease 1,10-phenanthroline-copper

complex to cleave the kinetoplast DNA to form linearized

kinetoplast DNA;

(c) purifying the linearized kinetoplast DNA to

form purified linearized kinetoplast DNA by:

10   (i) deproteinization with a phenol-

chloroform mixture;

(ii) ethanol precipitation with a glycogen

carrier; and

(iii) filtration through a

15 microconcentrator;

(d) amplifying the purified linearized

kinetoplast DNA by a sequence-specific method employing

at least two primers capable of hybridizing to conserved

regions of the catenated closed circular kinetoplast DNA

20 of T. cruzi; and

(e) detecting disease caused by T. cruzi by

identifying the presence of amplified DNA corresponding

to the primers and thus having sequences specific for

catenated closed circular kinetoplast DNA of T. cruzi by

25 hybridization to the primers.

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36. The method of claim 35 wherein the linearized DNA comprises fragments of from about 1 kb to about 1.5 kb in length.

5   37. The method of claim 34 wherein the sequence-specific amplification method is the polymerase chain reaction method.
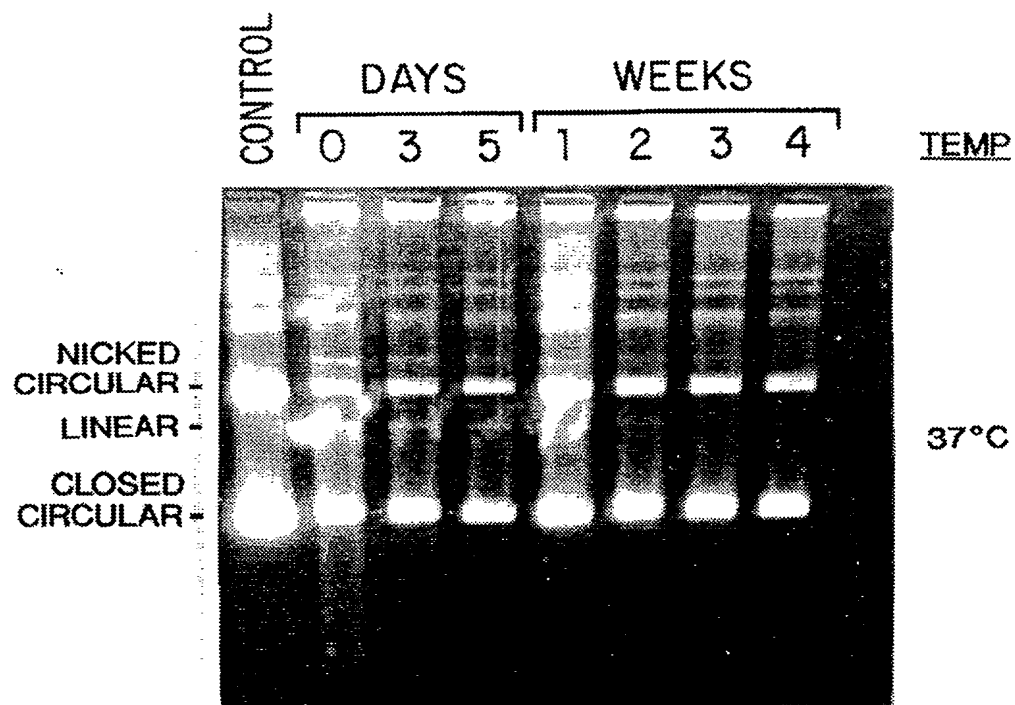
FIG. 1A
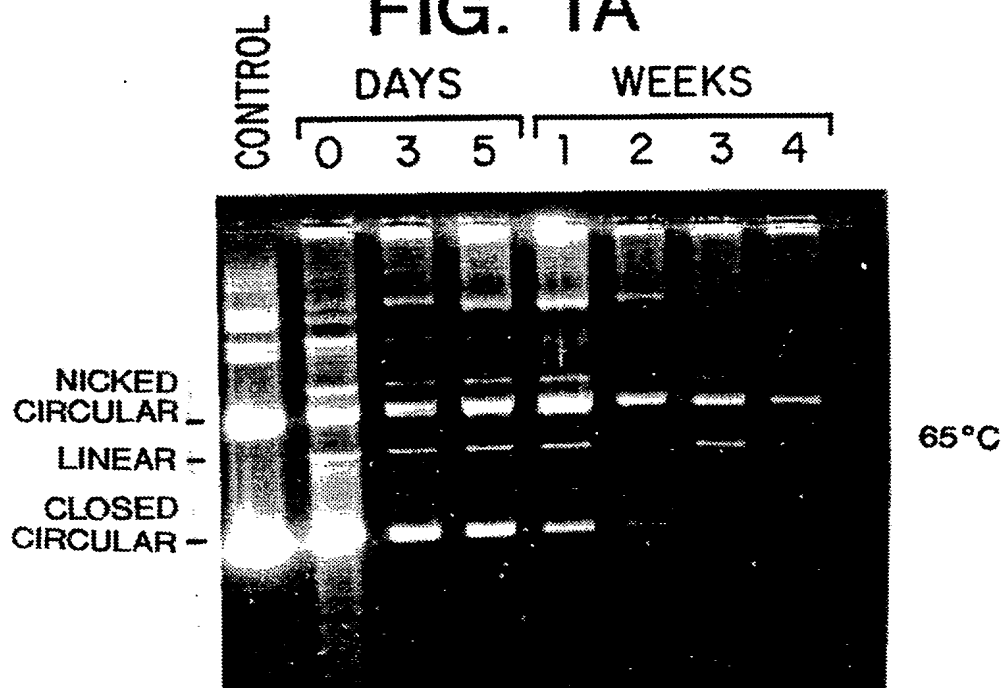
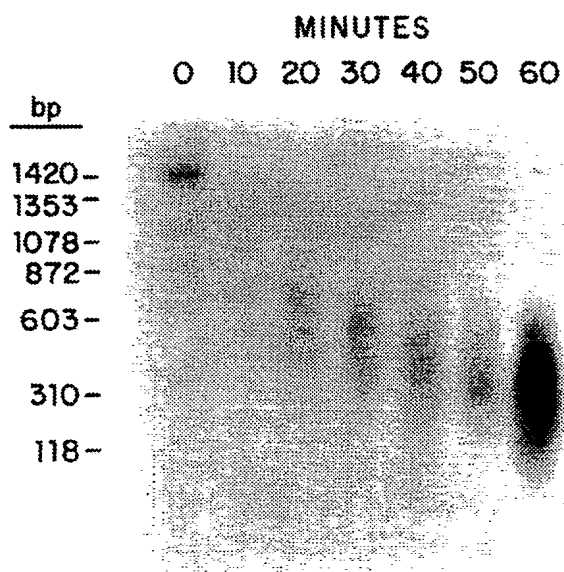FIG. 1B
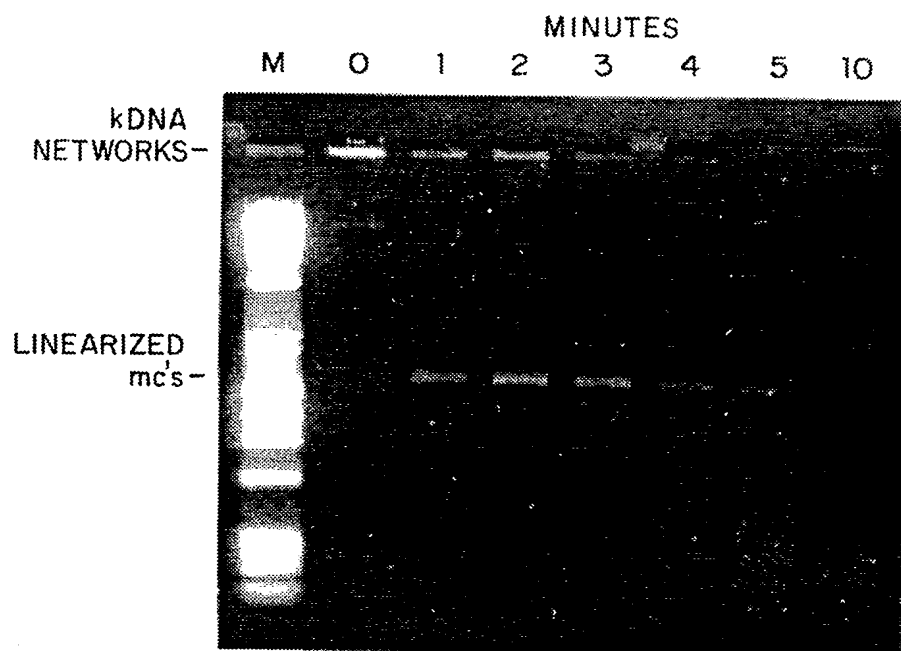
FIG. 2

FIG. 3

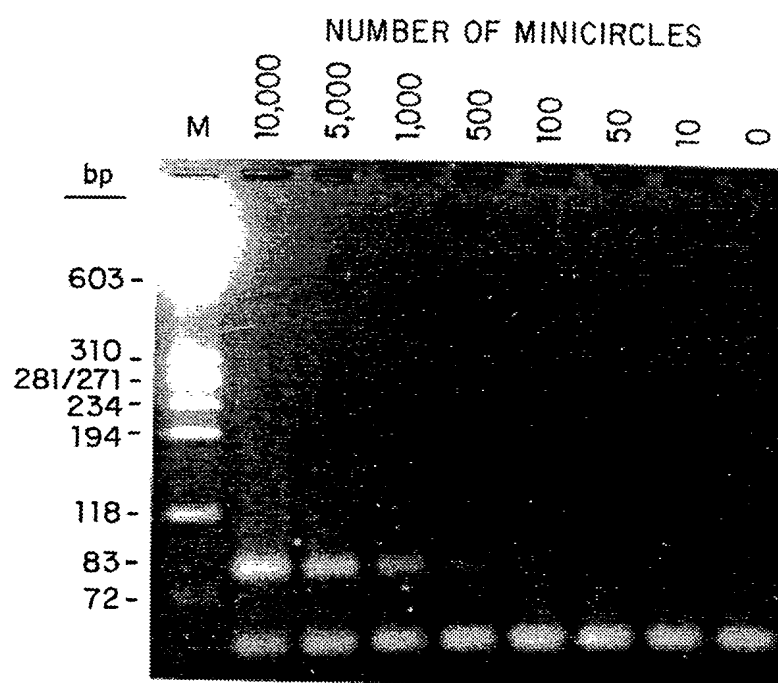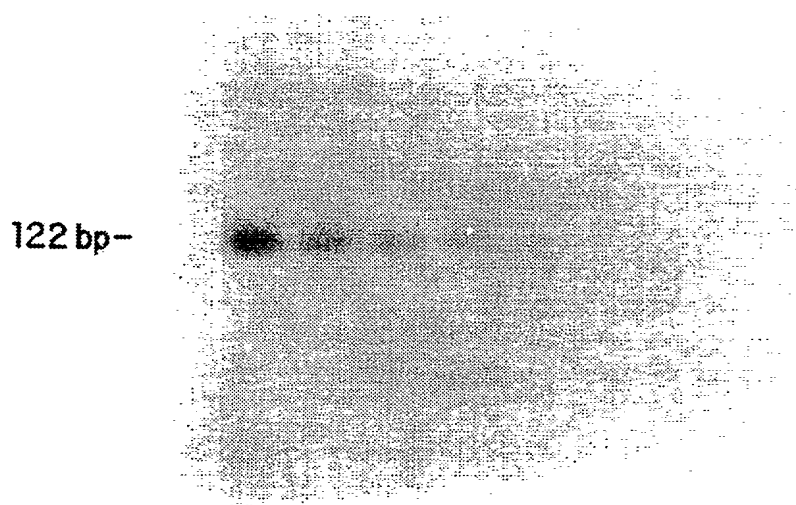FIG. 4A
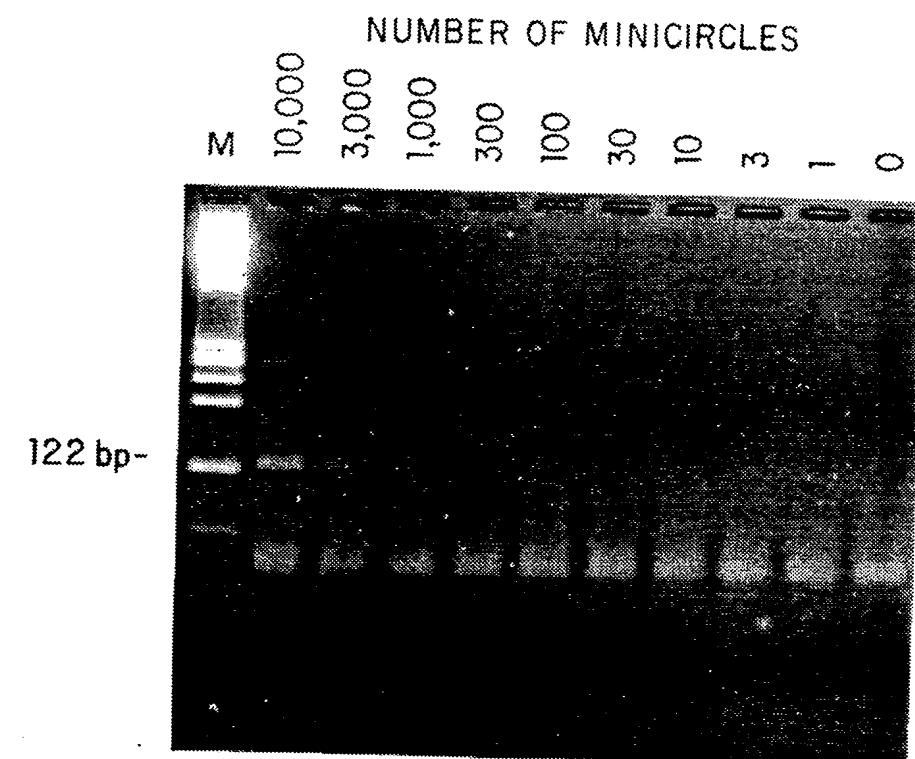
FIG. 4B
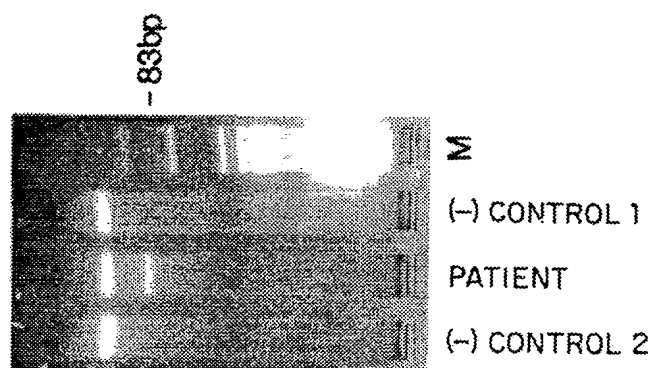
FIG. 5A
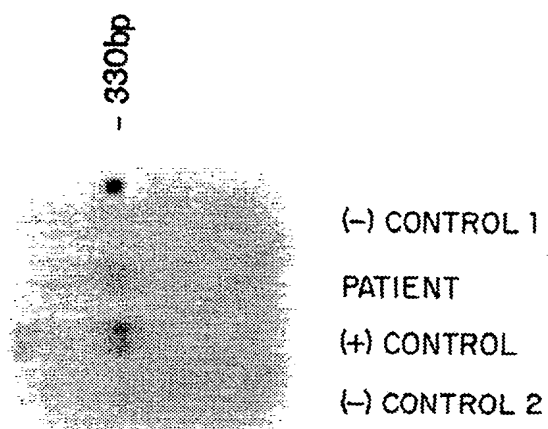
FIG. 5B
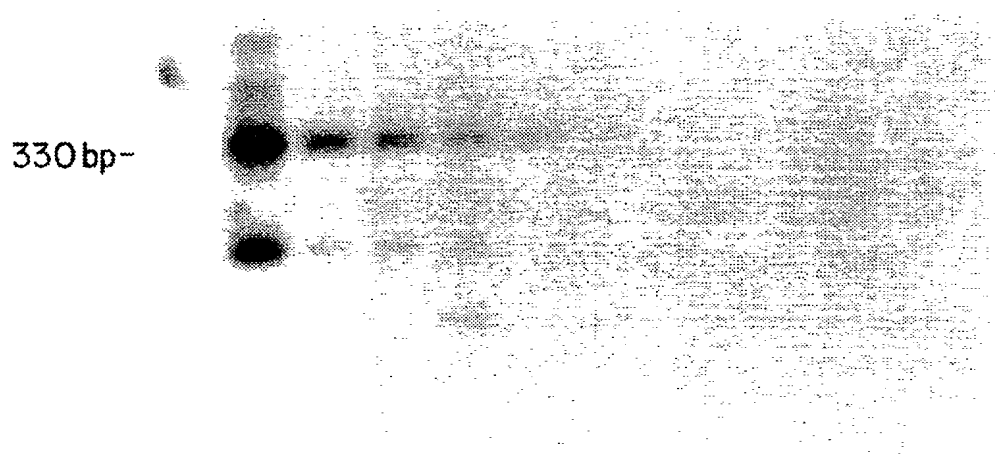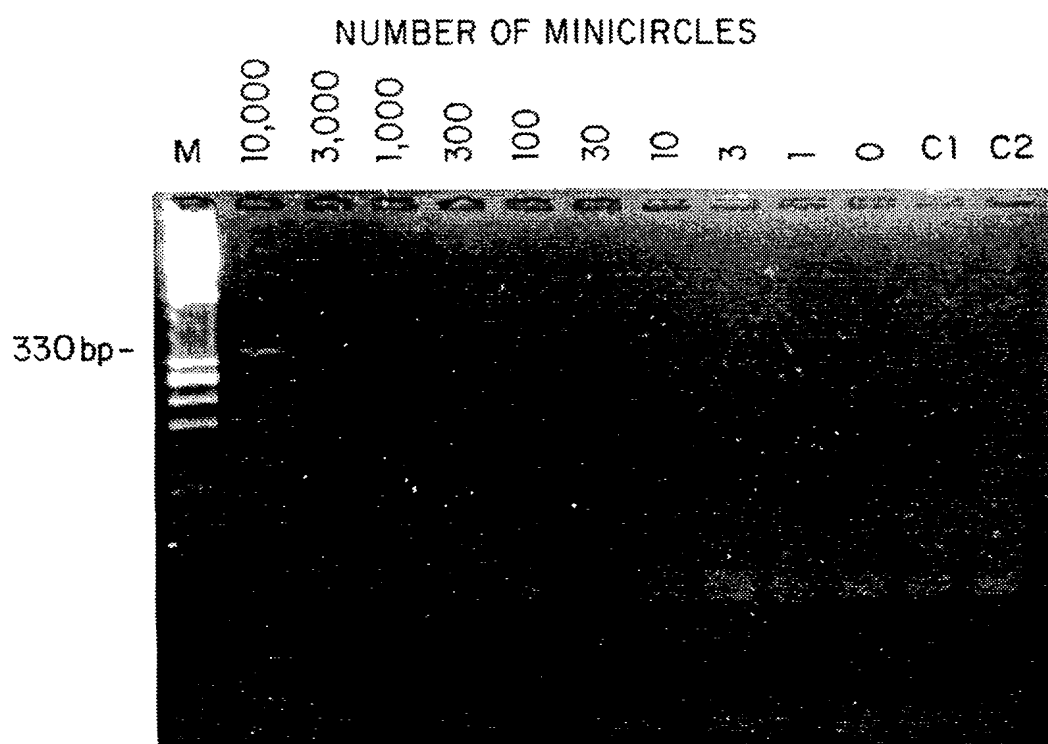
FIG. 5C
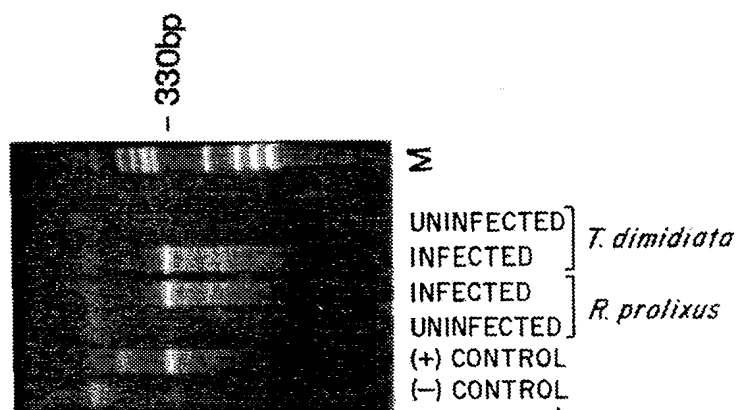
FIG. 6

INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :C12P 19/34; C12Q 1/68
US CL :435/91, 6

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/91, 6

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

CHEMICAL ABSTRACTS

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Molecular and Biochemical Parasitology, Volume 33, issued 1989, N. R. Sturm et al., "Sensitive detection and schizodeme classification of Trypanosoma cruzi cells by amplification of kinetoplast minicircle DNA sequences: use in diagnosis of Chagas' disease", pages 205-214, ENTIRE DOCUMENT.	1-37
Y	Experimental Parasitology, Volume 71, issued 1990, M. R. Rodgers et al., "Amplification of Kinetoplast DNA as a Tool in the Detection and Diagnosis of Leishmania", pages 267-275, especially page 273, paragraph bridging the left- and right-hand columns.	1-37
Y,P	US, A, 5,130,423 (Van Ness et al.) 14 July 1992, col. 3, lines 53-59.	1-37
Y	Annual Review of Biochemistry, Volume 59, issued 1990, D. S. Sigman et al., "CHEMICAL NUCLEASES: NEW REAGENTS IN MOLECULAR BIOLOGY", pages 207-236, entire document.	1-37

☐ Further documents are listed in the continuation of Box C.   ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be part of particular relevance		
"E"	earlier document published on or after the international filing date	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed	"&"	document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
26 SEPTEMBER 1992	01 OCT 1992

Name and mailing address of the ISA/ Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	Authorized officer JAMES KETTER
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